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Human plasma can modulate micronucleus frequency in TK6 and OE33 cells in vitro

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ABSTRACT

In this paper, we studied the potential genotoxic effects of human plasma from healthy volunteers, as well as patients with gastro-oesophageal reflux disease, Barrett's oesophagus (BO) and oesophageal adenocarcinoma (OAC) using the oesophageal adenocarcinoma cell line (OE33) and the lymphoblastoid cell line (TK6). Both TK6 and OE33 cells were treated with plasma (10 % volume, replacing foetal bovine serum (FBS) or horse serum (HS)) at different time points of 4 h (for the micronucleus (Mn) assay and the invasion assay) and 24 h (for the cell cycle studies). Plasma-induced effects on DNA damage levels, cell viability and the cell cycle were studied by the micronucleus assay, cytokinesis block proliferation index (CBPI) and flow cytometry respectively. The expression of IL-8 in supernatants of TK6 cells and IFN-β in OE33 cells was also analysed by enzyme-linked immunosorbent assay (ELISA). Finally, we carried out an assessment of cellular invasion of OE33 cells following plasma treatment.

The results of the micronucleus assay confirmed the genotoxicity of direct plasma treatment from some participants through the increase in DNA damage in TK6 cells. Conversely, some individual patient plasma samples reduced background levels of TK6 cell Mn frequency, in an anti-genotoxic fashion. In TK6 cells, (on average) plasma samples from patients with Barrett's oesophagus induced higher micronucleus levels than healthy volunteers ($p = 0.0019$). There was little difference in Mn induction when using plasma versus serum to treat the cells *in vitro*. Cell cycle results showed that direct plasma treatment had a marked impact on OE33 cells at 24 h ($p=0.0182$ for BO and $p=0.0320$ for OAC) by decreasing the proportion of cells in the S phase, while plasma exposure was less impactful on the cell cycle of TK6 cells. Invasion of OE33 cells was also seen to be non-significantly affected by plasma treatment of OE33 cells.

The addition of N-acetyl cysteine NAC in a dose-dependent matter did not alter the formation of Mn in TK6 cells, suggesting that reactive oxygen species (ROS) are not the root cause of plasma's genotoxicity. The concentration of IL-8 in TK6 cells and IFN-β in OE33 cells was significantly higher in cells treated with OAC-derived plasma than in the untreated negative control. Collectively, our results demonstrate that plasma-specific effects are detectable which helps us better understand some important aspects of the biology of blood-based biomarkers under development.

1. Introduction

Plasma is the liquid component of whole blood and is characterised by its light-yellowish or straw-coloured appearance [29]. It is important to note that plasma does not contain erythrocytes (RBCs), leukocytes (WBCs), and thrombocytes (platelets) which are the solid components of blood. Comprising >90 % water, plasma is an essential component of blood. Also present in plasma are coagulants, mainly fibrinogen, which play a key role in blood clotting [13]. Serum, often confused with plasma, is plasma without these clotting factors ([14], Kamath & Lip, 2003). Plasma proteins, such as albumin and globulin, help maintain the colloidal osmotic pressure at approximately 25 mmHg [15,53]. Electrolytes such as sodium, potassium, bicarbonate, chloride, and calcium help regulate blood pH [24]. In addition to the above, plasma contains

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immunoglobulins which help to fight infections, along with smaller amounts of secreted enzymes, hormones, growth factors and vitamins [30]. Plasma also contains products shed by cells and picked up by blood perfusing through tissues, this can include small amounts of biomolecules including DNA. In previous studies, it was proposed that circulating nucleic acids in plasma and serum (CNAPS) could be utilized for personalized cancer diagnosis, tracking the progress of treatment, and predicting prognosis [20]. These nucleic acids are derived from various sources including (a) the breakdown of blood cells, (b) bacteria and viruses, (c) leukocyte surface DNA, (d) cell and tissue necrosis, (e) apoptotic release of nucleosomes, (f) cellular release of exosomes, (g) transposons and retro-transposons, and (h) spontaneous release of a newly synthesized DNA/RNA-lipoprotein complex from living cells, also known as "the virtosome". When they enter recipient cells, they can alter the biology of those cells [19].

Liquid biopsies (using blood-based biomarkers) have been proposed as a less-invasive tool for diagnosing diseases like cancer, hence understanding the contribution that plasma makes to a liquid biopsy approach is vital. One promising liquid biopsy approach to detect both cancer and non-cancer conditions is the lymphocyte micronucleus assay [17,18,6,8]. Chromosome damage is readily assessed in human lymphocytes and can be linked to human exposure to genotoxic agents and can also be elevated in specific diseases like cancer [11]. The micronucleus assay is also a key test system used *in vitro* and *in vivo* for detecting genotoxic chemicals. It identifies and quantifies the formation of small DNA fragments, called micronuclei, in the cytoplasm of interphase cells, post-mitosis [33]. These micronuclei can arise from acentric fragments or whole chromosomes that fail to migrate during cell division. Therefore, the assay can detect both clastogenic and aneugenic chemicals [42]. The cytokinesis-block methodology, which involves adding cytochalasin B, allows scientists to identify nuclei that have undergone one division as binucleates. This makes it possible to also study the mechanisms of micronucleus (Mn) induction by combining the cytokinesis-block method with immunochemical labelling of kinetochores or hybridization with centromeric/telomeric probes [16]. In order to assess lymphocyte Mn from human donors, lymphocytes are separated from whole blood and subject to growth stimulation (by phytohaemagglutinin (PHA)) for 72 h with cytochalasin B added for the last 24 h to encourage the formation of binucleate cells (Fenech, 2000).

It has been shown that some cancer patients have elevated levels of lymphocyte Mn compared to non-cancer controls [32,7]. Hence, this suggests that lymphocytes circulating through primary tumours may be exposed to genotoxic agents that subsequently lead to Mn formation *ex vivo* after PHA-stimulated cell growth. Alternatively, reactive compounds released into the blood by the tumour may cause genotoxic effects in circulating lymphocytes more distantly [3]. Here we aim to understand if the plasma component of blood from patients can induce genotoxicity, as measured by the Mn assay in cell lines *in vitro* and to specifically understand if ROS may be responsible for such genotoxicity.

TK6 is a human lymphoblastic cell line; OE33, is a cell line derived from the adenocarcinoma of the lower oesophagus (Barrett's metaplasia). These cell lines are relevant to our research as they provide insight into the cellular composition and behaviour of this disease.

We have recently been interested in measuring Mn levels in patients with oesophageal disease and identifying the causes of this chromosome damage within lymphocytes. This would allow us to assess any clinical correlations of this biomarker with patient characteristics which may aid in clinical decision-making. This paper aims to evaluate the potential effects of the patient-derived plasma on Mn formation in two cell lines and to assess the antioxidant effects of N-acetylcysteine on Mn formation *in vitro*. Previous studies have investigated the potential use of plasma itself as a biomarker for the diagnosis of oesophageal squamous cell carcinoma (ESCC). Particularly, plasma miR-31 and miR-375 levels have demonstrated high sensitivity and specificity for differentiating ESCC patients from healthy individuals [22]. However, there is little data in the literature on plasma-induced effects *in vitro,* such as Mn formation. Here we use the gastroesophageal reflux disease (GORD) model of oesophageal adenocarcinoma formation to study the effects of plasma.

GORD is a chronic digestive disorder that is characterized by the regurgitation of gastric contents into the oesophagus [54]. GORD is a frequently diagnosed condition in the United States, affecting roughly 20 % of the population and resulting in a significant economic burden, including both direct and indirect costs, as well as adverse effects on quality of life [10]. GORD can be caused by a variety of intrinsic and/or structural, mechanisms that can lead to the disruption of the barrier at the oesophago-gastric junction, exposing the oesophagus to acidic gastric contents. The most common symptoms of GORD are heartburn and regurgitation, but it can also present in an atypical manner with extra-oesophageal symptoms such as chest pain, dental erosions, chronic cough, laryngitis, or asthma [2,21]. While lifestyle modifications and proton pump inhibitors (PPIs) have traditionally been the mainstay in the management of GORD, there is an increasing prevalence of medically refractory GORD, which necessitates a tailored approach to the management of the condition [43].

Barrett's oesophagus (BO) is a condition that is commonly associated with chronic GORD, and it is the only known precursor to oesophageal adenocarcinoma, a highly lethal form of cancer that has been on the rise for the last 50 years [45]. Surveillance of patients with known BO, and the medical and endoscopic treatment of BO and its associated early neoplasia, are advised and revised guidelines have been put in place that utilize the Grading of Recommendations, Assessment, Development, and Evaluation methodology [37,45].

OAC is the sixth leading cause of cancer-related deaths worldwide [28]. The incidence rates of this cancer type vary in different geographical regions. In some areas, higher rates of oesophageal cancer may be due to increasing tobacco and alcohol use which leads to increasing GORD symptoms, certain nutritional habits, and obesity [25,49]. This cancer type is more common in men than women [46] and there has been some research investigating systemic hormonal differences that might explain this sex-specificity [12,47,55].

N-acetylcysteine (NAC)- is a compound that contains a sulfhydryl group donor, which serves as a precursor of glutathione synthesis and inhibits the formation of ROS [31]. It is currently being investigated in clinical trials and experimental models for various respiratory conditions, such as chronic obstructive pulmonary disease and idiopathic pulmonary fibrosis [48,9]. It can also be used in cases of acetaminophen poisoning and in experimental models of cirrhosis and portal hypertension [27,40,50].

In addition, N-acetylcysteine has been shown to be an antigenotoxic agent, exhibiting protective effects against oxidative DNA damage due to its antioxidant properties [38]. Furthermore, it may decrease cytogenetic damage produced by exposure to cigarette smoke and mutagenic agents such as urethane and 2-acetylaminofluorene in rodents, suggesting potential chemo-preventive effects [4]. We employ NAC here to specifically assess if supplementing the glutathione enzyme system can affect plasma-induced Mn levels, hence assessing a role for ROS.

2. Methods

2.1. Blood collection and separation

Blood samples were collected from patients with oesophageal disease (at Singleton Hospital) and from healthy volunteers (at Swansea University). Ethical approval was obtained for collecting blood samples from healthy volunteers (13/WA/0190), endoscopy patients (11/WA/0367), and oncology patients (17/NI/0055). Informed consent was obtained from all patients before blood collection. Samples were collected in EDTA-coated tubes (BD, US) for plasma isolation and stored at room temperature and processed on the same day as collection. Patient data were anonymized, and processed plasma/serum samples were stored at −80 °C. A concentration of 10 % was used for plasma and serum for all the assays.

2.2. Sample preparation

Mononuclear cells (MNCs) were isolated by layering whole blood (1:1 ratio) onto sterile Histopaque (density 1.077 g/mL) (Sigma-Aldrich; UK) prior to centrifugation at 805 x *g* for 20 min at room temperature. Centrifugation resulted in four distinct layers according to density; plasma, MNC layer, Histopaque and red blood cells combined with the polymorphonuclear cells (Fig. 1). The plasma layer (yellow colour) was carefully removed with a sterile plastic Pasteur pipette, centrifuged at 515 x *g* and stored at −80 °C. For serum isolation, blood was instead collected in Serum activator tubes (Greiner Bio One; Vacuette 9 mL Z serum clot activator; 455092) and stored at −80 °C.

TK6 and OE33 cells were isolated and diluted in media according to the required concentrations for seeding. Cell counts were performed using a Z1 Coulter Particle Counter by Beckman Coulter.

2.3. CBPI and Mn induction

The CBPI was used to estimate cell viability based on the average number of cell divisions completed by a cell population, revealing cytotoxic relationships with tested substances. Cytotoxic compounds are expected to lower the average number of cell divisions. To calculate the CBPI, the formula CBPI = (MI + 2MII + 3(MIII + MIV))/total can be used, where MI-IV indicates the number of cells with one to four nuclei (Surrallés et al., 1995).

Binucleated cells were scored for the presence of Mn here based on certain characteristics such as the two nuclei having intact nuclear membranes and residing within the same cytoplasmic boundary. Mn were scored based on their diameter, shape, refractivity, and staining intensity, among other factors based on the criteria set out by Fenech (Fenech et al., 2003).

For CBPI, TK6 and OE33 cells were seeded at a density of 1.5×10^5 cells per mL with a volume of 3 mL in 6-well plates and incubated for 24 h. Among the six wells, one served as a control containing serum-free media (without horse serum (HS) for TK6 cells and foetal bovine serum (FBS) for OE33 cells respectively). After 4 h of plasma treatment, cells were re-seeded in complete media with the addition of 4.5μg/mL of cytoB. Cells were harvested 24 h later onto microscope slides for manual CBPI analysis. Where cells were fixed for 10 min in 90 % methanol (Fisher Chemical) and stained with 20 % Giemsa's stain (VWR Chemicals) in phosphate buffer M/15 (pH 6.8) reagent (Thermo Fischer Scientific, UK). Cells were stained for 10 min, before being washed 3 times with 6.8 pH phosphate buffer. Giemsa-stained cells were fixed once more using Xylene (Fisher Scientific), before DPX mounting medium (D/5330/05), Fisher Scientific) was used to fix coverslips to the slides. Slides were stored at 4 °C in the dark prior to analysis.

For the Mn assay, a semi-automated Metafer™ image analysis method was used as described in [51].

2.4. Cell cycle analysis

TK6 and OE33 cells were seeded at a concentration of 1.5×10^5 cells mL^{-1} in 24-well plates, in a total volume of 1 mL per well. Cells were incubated for 24 h before the media was changed, and plasma samples (10 %) were added. At 24 h after treatment, replicates of cells were harvested and isolated. The supernatant was discarded after centrifuging again with 1 mL phosphate buffer saline (PBS) (ThermoFisher Scientific; USA) before resuspending in 400μL PBS cooled on ice. Resulting cell suspensions were transferred to 5 mL fluorescence-activated cell sorting (FACS) tubes (Falcon) and vortexed whilst 800μL 100 % ethanol (Fisher Chemicals) was added dropwise. Fixed cells were stored for a minimum of 2 h at 4°C. Suspensions were centrifuged to isolate the cell pellet and washed with 2 mL PBS. After the PBS was discarded, 200μL FxCycle PI/RNAse staining solution (Invitrogen) was added to each tube and left in the dark at room temperature for 30 min. Cells were vortexed gently before processing with a Novocyte flow cytometer system (Agilent) (Fig. 2).

2.5. Invasion of OE33 cells treated with plasma and serum

OE33 cells were seeded at 5×10^4 cells/mL in 24-well plates in a total volume of 1 mL and incubated for 24 h. The cells were harvested and isolated whilst fresh wells were prepared to a total volume of 600μL, with treatment wells containing 10 % plasma or serum. Millicell cell culture transwells (Merck Millipore) were placed into the wells before isolated cells were resuspended in 400μL media-FBS and placed into the transwells. After 4 h of incubation, the media was removed from the transwells, and they were placed into fresh wells containing 500μL 90 % methanol for 10 min to fix the cells. Following this, the methanol was exchanged for 500μL 20 % Giemsa's stain (VWR Chemicals, USA) for 12 min. The transwells were washed three times in pH 6.8 staining buffer before images were taken at 4x magnification using an EVOS XL Core cell imaging system (Thermo Fisher). Using ImageJ, images were adjusted with a colour threshold to target the Giemsa-stained cell colonies present on the underside of the transwell. The area of the invaded cells was then expressed as a percentage of the total area of the image.

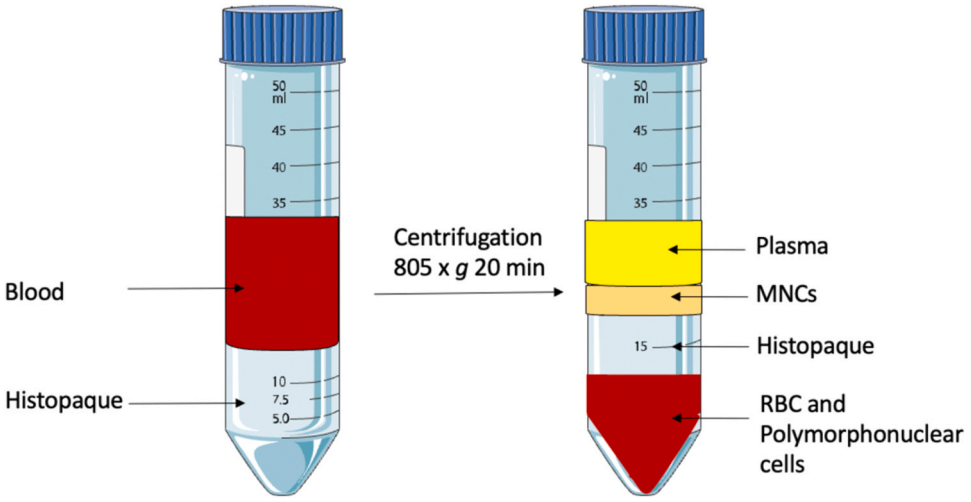

Figure 1. Plasma isolation from whole blood. Human blood was layered onto a Histopaque gradient at a 1:1 ratio. Density gradient centrifugation separation resulted in four distinct layers: plasma, mononuclear cells (MNC), Histopaque, and red blood cells (RBC)/polymorphonuclear cells. Figure made using BioRender.

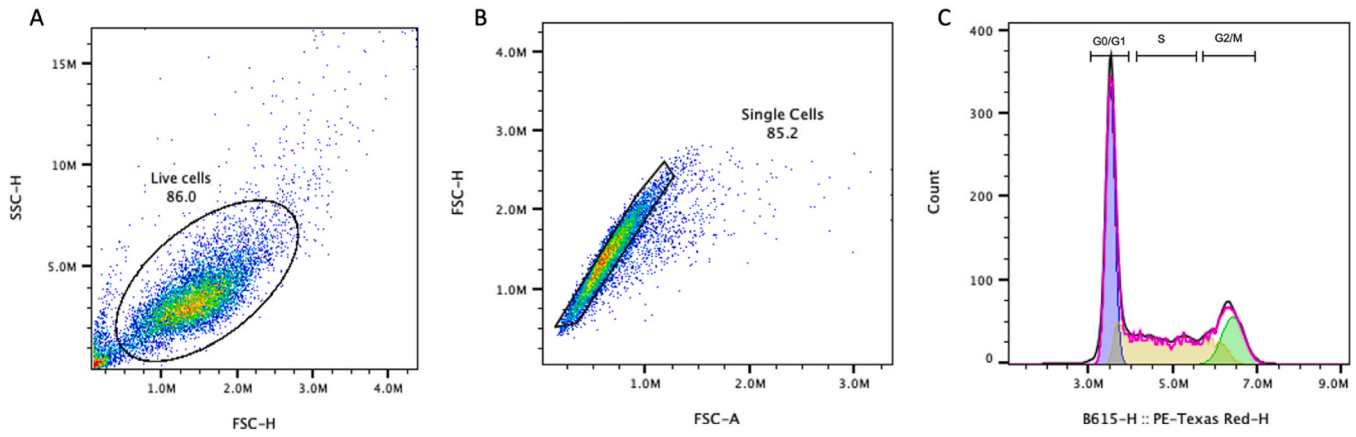

Figure 2. Example of the gating strategy for cell cycle analysis**.** An oval gate was applied to exclude debris and encompass live cells (A). To isolate the single cells, FSC-A versus FSC-H was applied, and a gate drawn around the single cells (B). A Cell Cycle Plot was applied to the single cells (C); each cell cycle stage was measured as a percentage of the total number of cells acquired.

2.6. Sandwich enzyme-linked immunosorbent assay

During the treatment of TK6 and OE33 cells for Mn and CBPI scoring, cell supernatants were collected and frozen at −20 °C. The supernatants were taken from the cells after 4 h of plasma treatment, just prior to the addition of cytoB. A sandwich ELISA was used here according to the manufacturer's instructions (R&D Systems Duo-Set; Bio-Techne). Initially, the ELISA plate was coated with the capture antibody that is specific for the targeted protein. The antibody was diluted to a working concentration in PBS and 50μL was added to each well of half area 96-well plates (Greiner Bio-One; Stonehouse; UK). The coated plate was left overnight at 4 °C. The next day, the excess capture antibody was discarded, and the remaining protein binding sites on the plate were blocked with 150μL of blocking buffer that consisted of 1 % BSA (Sigma-Aldrich; Poole; UK) dissolved in PBS. The plate was incubated in the block buffer for one hour at room temperature with gentle agitation on a Heidolph Titramax 1000 plate shaker (Heidolph instrument; Schwabach; Germany) at 450 RPM. The blocking buffer was discarded, and the plate was washed three times with 200μL/well of washing buffer (0.05 % of Tween 20 (Sigma-Aldrich; Poole; UK) in PBS). The detection antibody was added to the plate for two hours at room temperature with gentle agitation. A working concentration of streptavidin-horseradish peroxidase (HRP) was applied to the plate for exactly 20 min at room temperature with gentle agitation. A 1:1 solution of hydrogen peroxide and tetramethylbenzidine (TMB; BD Biosciences) was prepared, and 50μL of the solution was applied to each well of the plate. A blue colour was allowed to develop in the dark at room temperature. A final 50μL of 1 M sulphuric acid (H_2SO_4) was added to each well, turning the blue solution into a yellow solution. The absorbance of the plate was measured at 450 nm using a plate reader (POLARstar Omega, BMG, Germany), and the final protein concentration was calculated using the standard curve using Excel Version 16.70 (Microsoft, USA).

2.7. Data analysis

Statistical analysis was performed using GraphPad version 9 (La Jolla, USA). Data are represented as the mean ± standard error of the mean (SEM) unless otherwise stated. Analysis of variance (ANOVA) was used to compare two or more group means with one variable (one-way ANOVA). For multiple comparisons, a two-way ANOVA or three-way ANOVA was used. All experiments have a replicate sample size of at least n=3, and significant values were taken as $p < 0.05$ graphically denoted as * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and ****$p \leq 0.0001$.

3. Results

3.1. Mn frequencies and CBPI score in TK6 and OE33 cells upon plasma treatment

Mn frequencies were assessed in TK6 cells after treatments with individual patient plasma samples (Fig. 3A). The frequency of Mn was measured using a semi-automated Metafer system (Zeiss) by analysing slides that contained cells treated with plasma or serum samples for four hours (plus 24 h recovery). In TK6 cells (77 samples used in total), the average fold increase in Mn % was significantly elevated upon treatment with plasma acquired from BO patients ($p= 0.0019$) compared to that from healthy volunteers (Fig. 3B). There was however a noticeable variability in the Mn % induced by different plasma samples from different participants. In some cases, the plasma treatment reduced the background Mn % in TK6 cells. For example, the Mn % fold change ranged from a decrease of 0.4333-fold of the untreated TK6 control, to an increase of 5-fold in TK6 cells. This observation suggests a complex genotoxic/antigenotoxic effect which is person-specific. Additionally, we investigated the effects of donor age and gender on the production of Mn in TK6 cells by plasma samples. There was no effect of age ($p=0.4631$) nor gender ($p=0.3514$) on Mn production in TK6 cells.

Mn frequencies were also assessed in OE33 cells after individual treatments with patient plasma samples. In OE33 cells (26 samples used in total), the average fold change in Mn % was slightly elevated upon treatment with plasma obtained from BO and OAC patients compared to healthy volunteers However, this increase was not significant ($p= 0.4302$ for BO; $p= 0.4939$ for OAC) (Fig. 4B). There was a noted variability in the Mn % induced by different plasma samples from individuals. Similarly to TK6, in some cases, the plasma reduced the background Mn % in OE33 cells. For example, the Mn fold change in plasma-treated cells varied substantially over the background level and varied from a 0.62-fold decrease to 1.79-fold increase in OE33 cells. This observation also suggests a complex genotoxic/antigenotoxic effect in OE33 cells which is patient-specific. There was no effect of donor age ($p=0.2854$) nor gender ($p=0.3956$) on Mn production in OE33 cells by plasma.

The CBPI score for TK6 cells (37 samples used) and OE33 cells (28 samples used) following plasma treatment were unaffected upon treatment with plasma obtained from healthy volunteers and patients (Fig. 5). Variations in CBPI were evident on an individual basis. For example, the maximum range in CBPI varied from 1.7 to 2.1 in TK6 cells and 1.2–1.56 in OE33 cells.

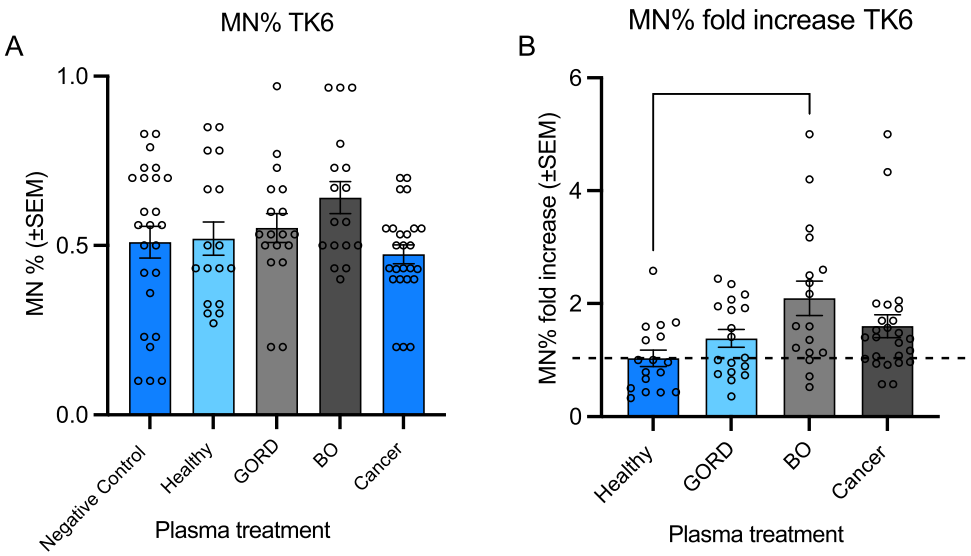

Figure 3. Mn score in TK6 cells treated with plasma**.** TK6 Cells were treated for 4 h with plasma from healthy volunteers or GORD, BO, and OAC patients (with 24 h further incubation with cytoB). (A) Mn frequency measurements in controls and cells treated with different plasma samples. (B) Mn frequency fold changes in cells treated with different plasma samples, relative to the control (77 samples used in total). Dotted line represents 1-fold (or no change). The experiments have a replicate sample size of at least n=3 for each group. Bar graphs represent means +/- SEM; and significant values were taken as $p < 0.05$ graphically denoted as * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$. GORD= Gastroesophageal reflux disease, BO= Barrett's Oesophagus.

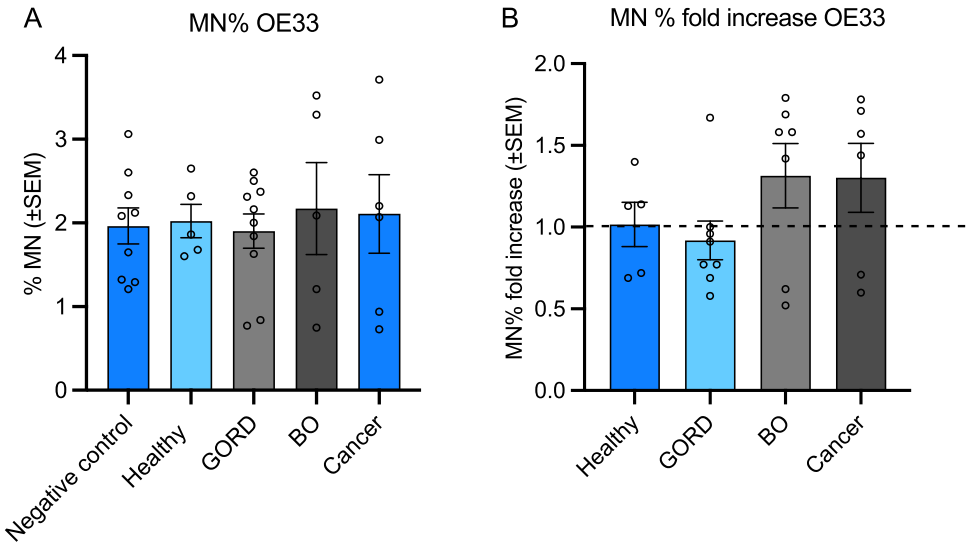

Figure 4. Mn frequency in OE33 cells treated with plasma**.** OE33 Cells were treated for 4 h with either healthy plasma, or plasma from GORD, BO, and OAC patients, followed by a further 24 h incubation with cytoB. (A) Mn frequency measurements in controls and cells treated with different plasma samples. (B) Mn frequency fold changes in cells treated with different plasma samples, relative to the control (26 samples used in total). Bar graphs represent means +/- SEM. The experiments have a replicate sample size of at least n=3 for each group. GORD= Gastroesophageal reflux disease, BO= Barrett's Oesophagus.

3.2. Plasma vs serum

Using a subset of samples, we compared the effect of plasma v serum, isolated from the same healthy individuals, in respect to Mn induction in TK6 and OE33 cells. When the cells were treated with serum from healthy individuals, it produced a non-significant change in Mn frequency in both cell lines (TK6 and OE33) compared to the untreated control cultured in serum-free media (as depicted in Fig. 6). Upon calculating the fold changes in Mn frequency from cells treated with healthy plasma and serum, TK6 cells (6 samples used in total) showed a non-significant increase in Mn frequency after serum treatment compared to plasma treatment whereas OE33 cells (11 samples used in total) showed very little difference between serum and plasma relative to their respective controls.

3.3. NAC

In order to investigate if the observed Mn induction by specific plasma samples obtained from participants was linked to circulating ROS, an antioxidant scavenger was employed pre-plasma exposure in TK6 cells. NAC at doses of 1, 10 and 50 mM were added to TK6 cells for 2 h before treatment with 10 % of the plasma (as above). As can be seen in Fig. 7, NAC pre-treatment did not reduce the Mn % compared to the same plasma samples treated alone. In fact, in some cases, NAC increased the fold change in Mn relative to the sample that was not treated with NAC (non-significantly). This was true for all the histological diagnoses. Hence, this suggests that plasma-associated ROS may not be the cause of the Mn induction by individual plasma samples (12 samples used) (Fig. 9). The CBPI scores for TK6 cells treated with the

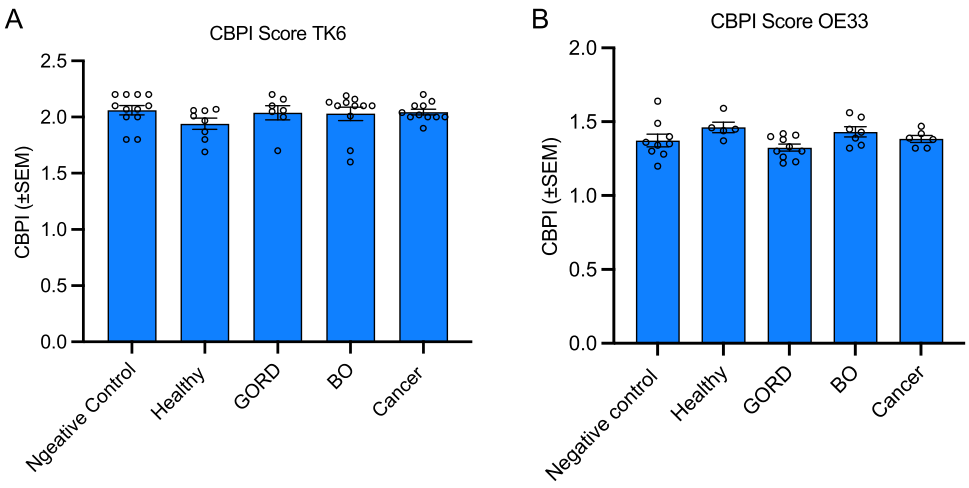

Figure 5. CBPI score in TK6 and OE33 cells treated with plasma**.** TK6 (37 samples used) and OE33 (28 samples used) cells were treated for 4 h with either healthy plasma, or plasma from GORD, BO, and OAC patients followed by a further 24 h incubation with cytoB. A) CBPI score in TK6 controls and cells treated with different plasma samples. B) CBPI score in OE33 controls and cells treated with different plasma samples. Bar graphs represent means +/- SEM. The experiments have a replicate sample size of at least n=3 for each group. GORD= Gastroesophageal reflux disease, BO= Barrett's Oesophagus.

different doses of NAC were also determined using manual scoring techniques (12 samples used). Fig. 9 illustrates the CBPI scores for all three doses of NAC (1, 10 and 50 mM). There was no clear effect of NAC pre-treatment on CBPI and hence on cell proliferation.

3.4. Cell cycle analysis of TK6 and OE33 cells treated with plasma

Cell cycle analysis was conducted on cells treated with plasma obtained from healthy individuals and from patients with GORD, BO, or cancer, using a Novocyte flow cytometer and the DNA stain PI (Fig. 8). Treatment of TK6 cells with plasma (30 samples used) for 24 h had no significant effect on the distribution of the cell cycle phases. However, OE33 cells (28 samples used) treated with plasma derived from BO and cancer patients showed a significantly lower proportion of the S phase cells compared to the negative control suggestive of an effect on cell proliferation and cell cycle progression (p= 0.0182 for BO; p= 0.0320 for OAC). This was accompanied by a non-significant increase in the G2/M phase (p= 0.0733 for BO; p= 0.1214 for OAC).

3.5. Plasma-induced invasion of OE33 cells

We measured the percentage coverage of invasion images using a colour thresholding function on ImageJ (Supplementary Figure 1). It was observed that the action of invasive cells slightly increased upon treatment with healthy serum, as compared to the control (Supplementary Figure 2A). Additionally, treatment with GORD, BO, and OAC plasma also appeared to increase the number of invaded cells compared to the control However, this increase was not significant (Supplementary Figure 2B).

3.6. Measurement of inflammatory cytokine content in supernatants from OE33 cells

Supernatants from cells treated with different plasma samples were used to measure the concentration of supernatant IL-8 and IFN-β using an ELISA method. Treatment with healthy volunteer plasma and plasma from cancer patients resulted in an increase in the concentration of IL-8 in TK6 cells (22 samples used) (p= 0.0303 for healthy; p= 0.0148 for OAC) (Fig. 9A). Similarly, the concentration of IFN-β was elevated in OE33 cells (17 samples used) after treatment with cancer patient plasma (p= 0.0479 for OAC). (Fig. 9B).

4. Discussion

Oesophageal cancer is a type of cancer that is among the 10 most common types of cancer in the world [34]. Unfortunately, oesophageal cancer may not show any visible symptoms until the cancer has spread [41] and this late presentation is the reason why survival rates are low.

To treat early-stage oesophageal cancer, healthcare providers typically rely on surgery to remove the tumours or alleviate symptoms [36]. In cases of more advanced or later-stage oesophageal cancer, they may use treatments such as chemotherapy, radiation therapy, and immunotherapy. When the cancer is not curable, healthcare providers focus on helping patients live longer while minimizing discomfort and maintaining their quality of life [5].

Scientists are actively working on developing new treatments to help people with oesophageal cancer live longer and improve their quality of life [23]. Oesophageal adenocarcinoma is linked to chronic reflux disease and with the condition Barrett's oesophagus. Surveillance programs aim to identify these precursor lesions and tailor follow-up to identify early cancers when they are more treatable. Understanding the biochemical changes in this histological progression series is valuable and may lead to the identification of biomarkers that may be linked to disease progression and be clinically useful. Here, we are interested in the role that lymphocyte micronucleus formation may play in disease aetiology and specifically if the plasma compartment of blood carries genotoxic compounds that may help explain the increased levels of lymphocyte Mn in oesophageal cancer patients.

DNA damage can be an early indicator of carcinogenic risk, although it is important to note that structural DNA damage may not always result in functional phenotypic effects [44]. Additionally, the DNA damage may not result in changes in protein expression, structure or function. However, if the DNA damage surpasses the cellular repair capacities, it may lead to genome instability or trigger the apoptotic pathway leading to cell death, ensuring that the damage is not propagated during future cell divisions [1]. The presence of DNA damage in the form of lymphocyte Mn can help to understand the biology of oesophageal carcinogenesis and may identify causative genotoxic exposures or predispositions which may help to better understand this type of cancer.

Circulating cell-free DNA (cirDNA) is a type of DNA that is released into the bloodstream by cells undergoing apoptosis. This phenomenon is linked to inflammation and cell damage [56]. The use of cirDNA detected in plasma and serum as a biomarker has gained significant attention in recent years due to its potential to serve as a non-invasive diagnostic tool for a range of diseases. However, the mechanisms

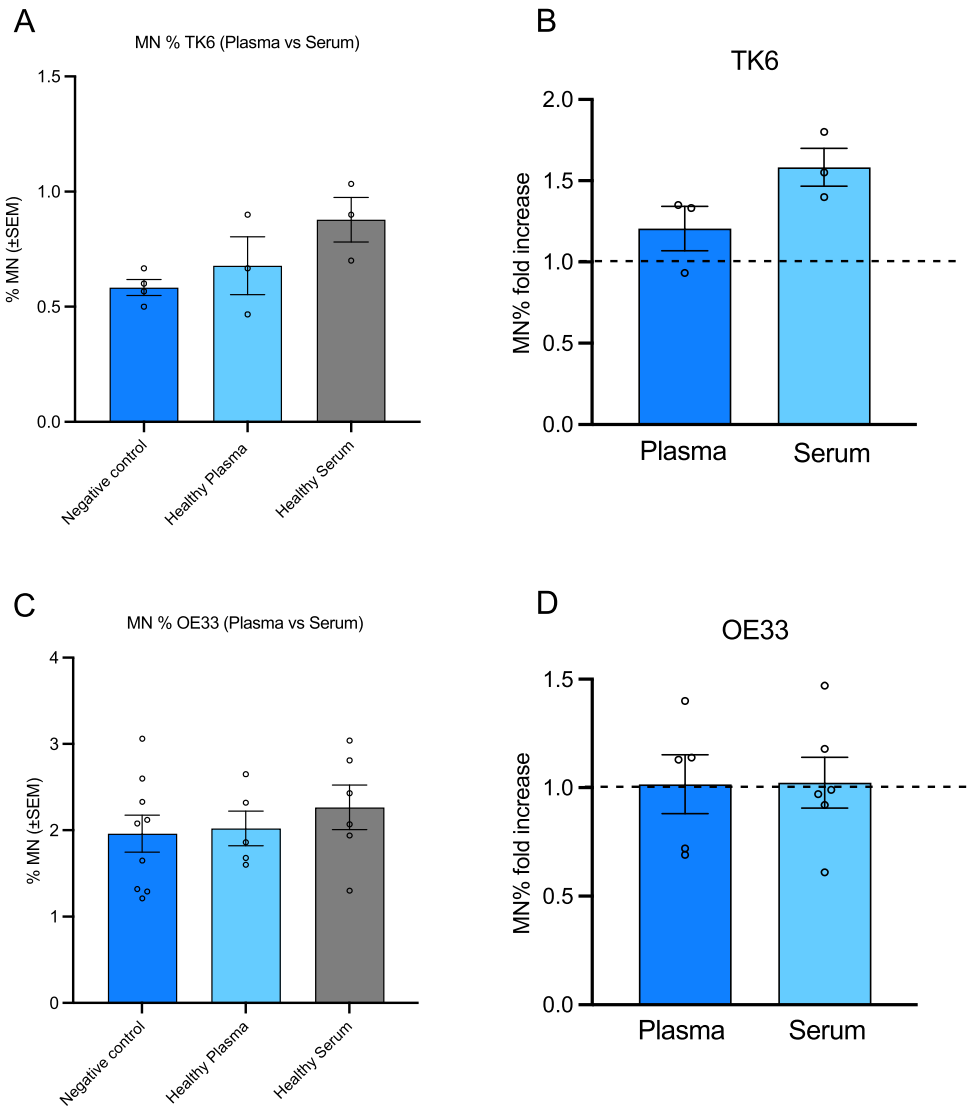

Figure 6. Mn frequency in TK6 and OE33 cells treated with plasma and serum derived from healthy volunteers. (A) Mn frequency in control and healthy plasma/serum-treated cells of TK6 cells. (B) Mn frequency fold change in TK6 cells treated with healthy plasma and serum, relative to the control (6 samples used in total). (C) Mn frequency in control and healthy plasma and serum-treated cells of OE33 cells. (D) Mn frequency fold change in OE33 cells treated with healthy plasma and serum, relative to the control (11 samples used in total). Bar graphs represent means +/- SEM. The experiments have a replicate sample size of at least n=3 for each group.

underlying its release and its role in disease remain under investigation. Acute exercise was identified as a factor that increases the induction of cirDNA in plasma [39], which is linked to the production of ROS [52]. Here we showed that plasma from patients with GORD, BO and OAC used to treat TK6 and OE33 cells enabled the detection of the frequency of induced micronuclei and hence the determination of the DNA damage (the genotoxic potential) caused by the plasma. The results showed that plasma isolated from BO patients increased the frequency of micronuclei in TK6 cells significantly compared to other histologies (but this was not the case in OE33 cells). The addition of the antioxidant, NAC at various doses (1–50 mM), did not affect the observed genotoxic effect of the plasma treatment. Hence, this suggests that the cause of the genotoxicity of individual plasma samples is unlikely to be linked to ROS.

Interestingly, it was found that the level of DNA damage (Mn %) varied considerably depending on the specific plasma used and was hence participant-specific. This was manifested in the example that plasma from healthy donors caused from 0.43-fold to 2.58-fold Mn induction in TK6 cells and 0.69-fold to 1.4-fold induction in OE33 cells. This interesting observation suggests that the genotoxic potential of individual plasma samples is based on an underlying difference in the mix of genotoxic and antigenotoxic compounds in individual plasma samples. Given that some plasma samples reduced Mn levels in TK6 cells by around 2-fold (and slightly less in OE33 cells), it would be interesting to explore what elements present in the plasma may be affording that anti-genotoxic effect. This has important implications for assessing the personalised cancer risks associated with individual participants. Given the widespread use of blood in transfusions and the risks associated with such donations, this variation in genotoxic v antigenotoxic properties of individual plasma may be worthy of follow-up. Finally, there are wider implications related to the understanding of plasma composition and its underlying biology when considering the use of blood-based biomarkers (liquid biopsies) in general.

Another interesting finding of the study is the elevated levels of IFN-β in the supernatant of OE33 cells treated with cancer-derived plasma. The cyclic GMP-AMP synthase cGAS is a DNA sensor that triggers an immune response by producing (cGAMP) to activate the stimulator of interferon genes STING. It can be activated by double-stranded DNA, including self-DNA, and genomic DNA damage (including Mn) and can lead to cGAS activation and inflammation [26]. The STING pathway is crucial in inducing IFN-β production by detecting pathogen-associated

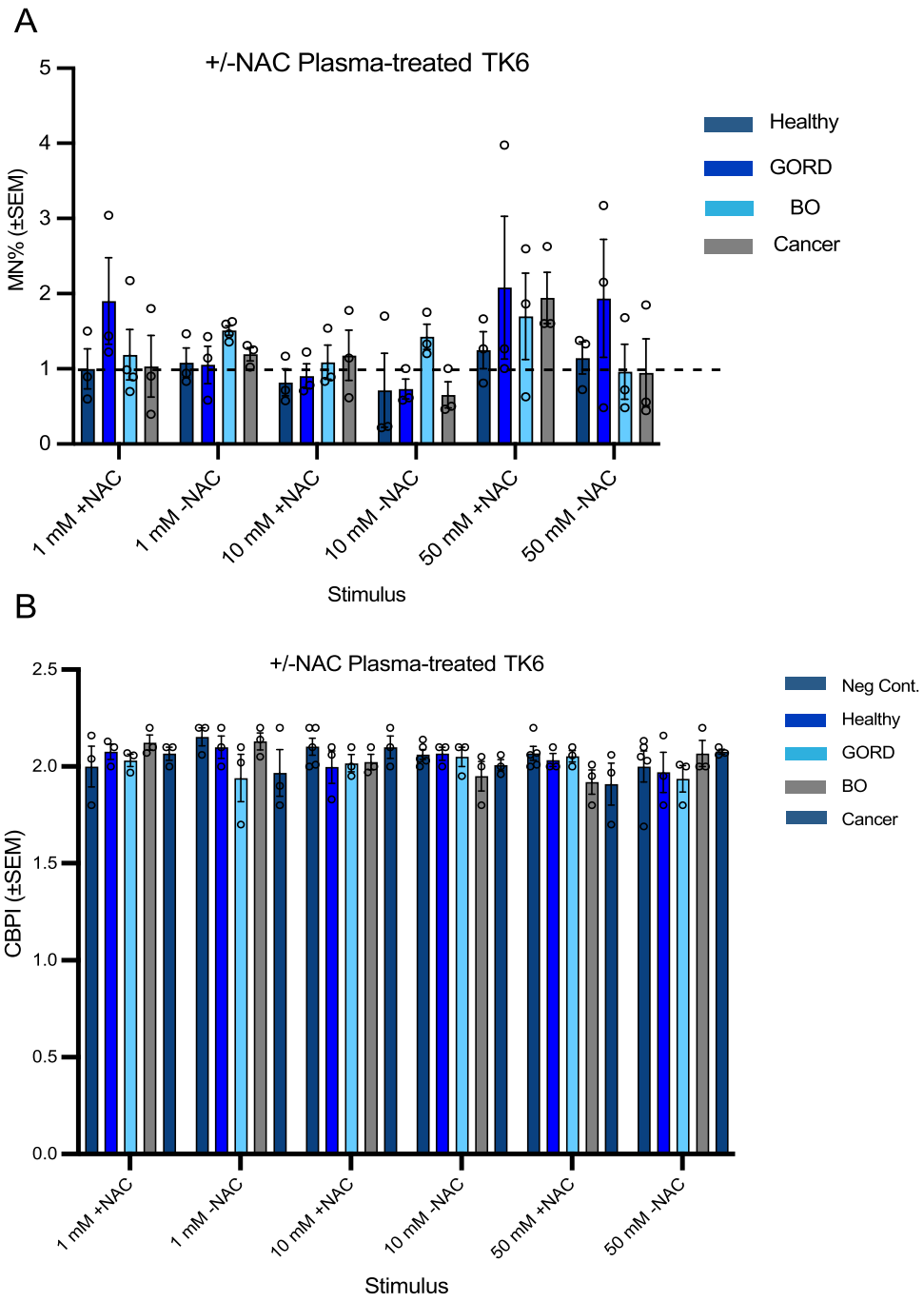

Figure 7. Mn % (A) and the CBPI scores (B) compared to the untreated negative control in TK6 cells after dosing with 1, 10 and 50 mM of NAC prior to being treated with plasma (4 h + 24 h). A total of 12 samples were used. Bar graphs represent means +/- SEM. The experiments have a replicate sample size of at least n=3 for each group. Gastroesophageal reflux disease, BO= Barrett's Oesophagus.

molecular pattern molecules (PAMPs)-like virus-derived nucleic acids. cGAS produces 2′3′-cGAMP, which binds to STING and induces IFN-β production [35]. This pathway could be linked to Mn production observed in OE33 cells in this study.

5. Conclusion

The results of this study have suggested a potential genotoxic action of BO plasma in comparison to healthy volunteers. It appears that the frequency of micronucleus induction in TK6 cells and OE33 cells varies considerably based on the individual's plasma, whether it was derived from healthy controls or GORD, BO, or OAC patients. The supplementation with NAC two hours before the plasma treatment did not affect the micronucleus frequency in TK6 cells. Therefore, the genotoxic potential of plasma samples was not related to ROS, and the causative genotoxic compounds remain unknown.

CRediT authorship contribution statement

Ethan Grewal: Investigation, Formal analysis, Data curation. **Shareen Doak:** Supervision. **Lisa Williams:** Resources. **Gareth Jenkins:** Writing – review & editing, Writing – original draft, Supervision. **Kathryn Munn:** Writing – review & editing. **Hamsa Naser:** Writing – original draft, Methodology, Investigation, Formal analysis. **Rhiannon**

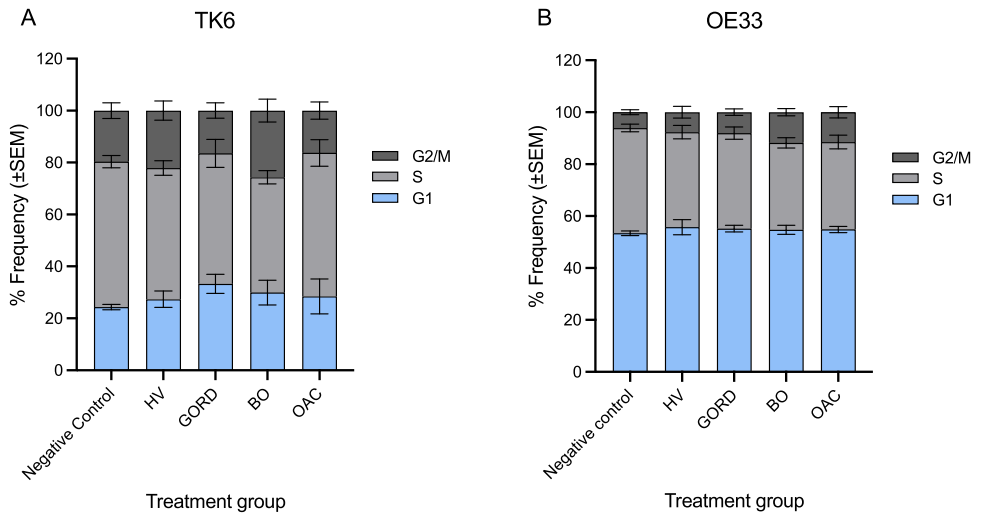

Figure 8. Frequencies of TK6 and OE33 cells treated with plasma in the different cell cycle phases**.** Cells were treated for 24 h with healthy plasma or plasma derived from GORD, BO and cancer patients. Cell cycle analysis was performed using a Novocyte flow cytometer. (A) Cell cycle phase frequencies in TK6 cells treated with plasma for 24 h (30 samples used in total). (B) Cell cycle phase frequencies in OE33 cells treated with plasma for 24 h (28 samples used in total). Bar graphs represent means +/- SEM; and significant values were taken as $p < 0.05$ graphically denoted as * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$. The experiments have a replicate sample size of at least n=3 for each group. GORD= Gastroesophageal reflux disease, BO= Barrett's Oesophagus, OAC= Oesophageal adenocarcinoma.

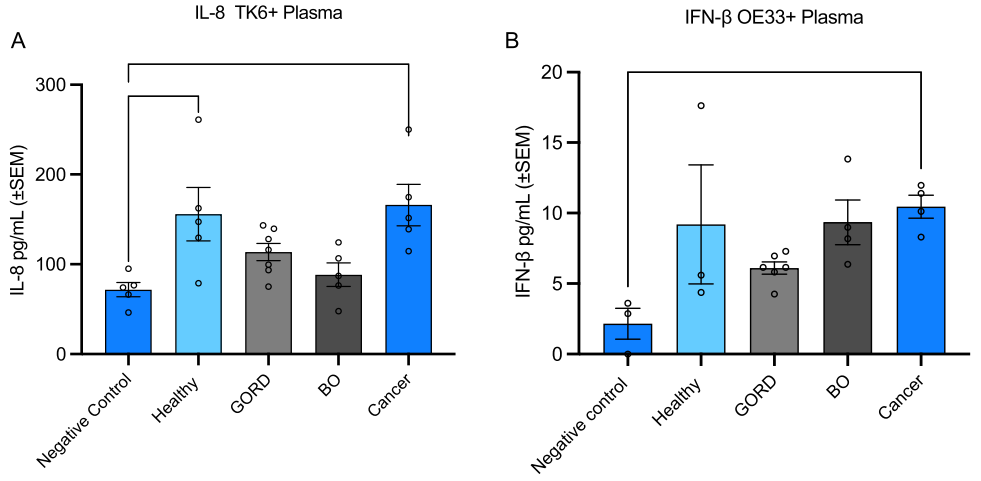

Figure 9. Results from an ELISA performed to measure IL-8 and IFN-β concentration**.** IL-8 concentration in plasma-treated TK6 supernatant (22 samples used in total) (A). IFN-β concentration in plasma-treated OE33 supernatant (17 samples used in total) (B). The experiments have a replicate sample size of at least n=3 for each group. Bar graphs represent means +/- SEM; and significant values were taken as $p < 0.05$ graphically denoted as * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$. GORD= Gastroesophageal reflux disease, BO= Barrett's Oesophagus.

Wright: Formal analysis, Data curation. **Rachel Lawrence:** Writing – review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.mrgentox.2024.503766.

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